

# Induction of Apoptosis in Mammalian Cells by Cadmium and Zinc

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In various mammalian cells, two group IIb metals, cadmium and zinc, induce several morphological and biochemical effects that are salient features of programmed cell death. In C6 rat glioma cells, cadmium caused externalization of phosphatidylserine, breakdown of the mitochondrial membrane potential, activation of caspase-9, internucleosomal DNA fragmentation, chromatin condensation, and nuclear fragmentation. In NIH3T3 murine fibroblasts, cadmium-induced apoptosis was inhibited by overexpression of the antiapoptotic protein Bcl-2. Cadmium-induced DNA fragmentation in C6 cells was independent of inhibition of protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase, Ca-calmodulin-dependent protein kinase, and protein kinase G. Zinc at moderate concentrations (10–50  $\mu\text{M}$ ) protected against programmed cell death induced by cadmium, whereas deprivation of zinc by the membrane-permeable chelator *N,N,N',N'*-terakis-(2-pyridylmethyl)ethylenediamine (TPEN) caused cell death with features characteristic of apoptosis. On the other hand, at elevated extracellular levels (150–200  $\mu\text{M}$ ), zinc alone caused programmed cell death in C6 cells. Zinc-induced apoptosis was independent of inhibition of PKA, PKC, guanylate cyclase and MAPK, but it was suppressed in the presence of 100  $\mu\text{M}$  lanthanum chloride. **Key words:** apoptosis, cadmium, DNA fragmentation, lanthanum, protein kinases, zinc. *Environ Health Perspect* 110(suppl 5):865–867 (2002).

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Cadmium is a multitarget toxicant for most organisms studied. In mammals it is nephrotoxic, neurotoxic, and carcinogenic (1). Cadmium can induce cell death either by necrotic or by apoptotic mechanisms. This metal was shown to induce apoptosis only in distinct cell types (2,3). In some instances, cadmium even protected from apoptosis caused by other agents (4). In contrast the essential element zinc is involved in the structure and function of many proteins in all organisms. Especially, zinc ions are integral parts of many enzymes and transcription factors controlling cell proliferation, differentiation, and cell death (5), and zinc ions therefore receive growing appreciation for their effects on the regulation of numerous cellular processes. A specific regulatory function of zinc has been demonstrated in the nervous system. Zinc ions have been shown to modulate the activity of NMDA (*N*-methyl-D-aspartate) receptors in the brain (6). Mobilization of zinc in toxic concentrations from synaptic vesicles is discussed as a cause of neuronal death in neural disorders (7,8). Among its other functions in cell proliferation, zinc protects against apoptosis induced by many agents, including cadmium (3,9,10), whereas zinc deprivation causes programmed cell death (11,12). However, zinc excess is cytotoxic (13) and, at least in some cell lines, can also induce apoptosis (12).

Cadmium and zinc ions interact with intracellular signal transduction on multiple levels. Cadmium has been shown to modulate second messenger metabolism, protein kinase

and phosphatase activities, and transcription factor binding to DNA (14). Cadmium (15) and zinc (16,17) evoked the mobilization of  $\text{Ca}^{2+}$  through the formation of inositol trisphosphate. Cadmium and zinc also increased intracellular levels of cyclic guanosine monophosphate (cGMP) by inhibiting the cyclic nucleotide phosphodiesterase (18). Furthermore, cadmium was shown to activate mitogen-activated protein kinase (MAPK) (19), protein kinase C (PKC) (20), and c-Jun kinase (21). The apparent increases in protein kinase activities are probably caused by inhibition of protein phosphatases, which are known to be caused by the heavy metals cadmium and zinc (22,23). Cadmium and zinc seem to be relatively specific inducers of apoptosis in C6 cells because the toxic metal ions  $\text{CrO}_4^{2-}$ ,  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Pb}^{2+}$  did not induce apoptotic DNA fragmentation (3).

## Induction of Apoptosis by Cadmium

Induction of apoptotic DNA fragmentation by cadmium had been observed in a few cell types so far, but it was not clear whether apoptosis represents a general mode of cell death in cells derived from different species and tissues. We investigated the eight mammalian cell lines listed in Table 1 and detected apoptotic DNA fragmentation in only three: C6 rat glioma cells, NIH3T3 murine fibroblasts, and E367 rat neuroblastoma cells, all tested in the range of cell-specific cytotoxic cadmium concentrations (Table 1). In C6 rat

glioma cells, cadmium produced the following salient features of programmed cell death (Table 2): externalization of phosphatidylserine from the inner to the outer side of the plasma membrane, breakdown of the mitochondrial membrane potential, activation of caspase-9, internucleosomal DNA fragmentation, chromosome condensation and nuclear fragmentation. In addition suppression of cadmium-induced apoptosis by overexpression of the antiapoptotic protein Bcl-2 was found in NIH3T3 mouse fibroblasts. The conditions of treatment are given in Table 2.

DNA fragmentation depended on cadmium concentrations and time, as shown in Figure 1. The cytotoxicity of cadmium in C6 cells is relatively high: in two independent viability assays 50% inhibition was produced by 0.7  $\mu\text{M}$   $\text{CdCl}_2$ . DNA fragment ladders appeared at a concentration of 1  $\mu\text{M}$   $\text{CdCl}_2$  and reached a maximum at 100  $\mu\text{M}$ ; at more elevated concentrations, necrotic cell death seems to prevail. In culture medium without serum, the concentration causing maximal DNA fragmentation was much lower: 10–15  $\mu\text{M}$   $\text{CdCl}_2$ . The induction of apoptosis by cadmium is a slow process: the DNA fragmentation appeared as late as 40 hr after addition of cadmium and reached its maximum at 48 hr.

Caspase-9 is a critical enzyme specific for programmed cell death. This enzyme is known to be activated after a breakdown of the mitochondrial membrane potential, formation of the permeability transition pore, and release of cytochrome *c* from mitochondria. Figure 2 shows that caspase-9 was activated in C6 cells by incubation with 5–10  $\mu\text{M}$   $\text{CdCl}_2$  for 48 hr. Combined with the observation that cadmium decreased the mitochondrial membrane potential in C6 cells, this result indicates that cadmium induces apoptosis via the mitochondrial pathway. Our result seems to contradict the observation of others who found that cadmium inhibited caspase-3,

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**Table 1.** Cell-type-specific induction of apoptotic DNA fragmentation by cadmium and zinc.<sup>a</sup>

Cell type	CdCl <sub>2</sub>		ZnCl <sub>2</sub>	
	LC <sub>50</sub> (μM)	DNA fragmentation	LC <sub>50</sub> (μM)	DNA fragmentation
C6 rat glioma cells	1	+	150	+
PC12 rat pheochromocytoma cells	15	-	375	-
E367 rat neuroblastoma cells	10	+	150	+
NIH3T3 mouse fibroblasts	10	+	70	-
L929 mouse fibroblasts	15	-	200	+
3T3L1 mouse preadipocytes	5	-	50	-
VH16 human fibroblasts	65	-	250	-
A549 human lung adenocarcinoma cells	160	-	600	+

<sup>a</sup>The indicated cell lines were incubated with different concentrations of CdCl<sub>2</sub> or ZnCl<sub>2</sub> for 48 hr to derive the LC<sub>50</sub> values that describe the metal concentrations where 50% of the cells were deficient in the neutral red uptake viability assay. Internucleosomal DNA fragmentation was analyzed after incubation at the respective LC<sub>50</sub> concentrations for 48 hr.

**Table 2.** Apoptotic features induced by cadmium and zinc in C6 glioma cells.<sup>a</sup>

Feature	CdCl <sub>2</sub>	ZnCl <sub>2</sub>
Cytotoxicity (EC <sub>50</sub> with neutral red uptake)	0.7 μM, 24 hr	155 μM, 24 hr
Internucleosomal DNA fragmentation	1 μM, 48 hr	150–200 μM, 48 hr
Chromatin condensation and nuclear fragmentation	2.5 μM, 48 hr	200 μM, 24 hr
Breakdown of the mitochondrial membrane potential	1 μM, 48 hr	200 μM, 6–24 hr
Externalization of phosphatidylserine	2.5 μM, 48 hr	Not done
Activation of caspase-9	5–10 μM, 48 hr	Not done
Plasma membrane blebbing	2.5 μM, 48 hr	200 μM, 6–9 hr
Suppression of DNA fragmentation by 100 μM La <sup>3+</sup>	No effect	Suppression
Suppression of DNA fragmentation by Bcl-2	Suppression	Not done

<sup>a</sup>Effects were measured in C6 rat glioma cells with the exception of susceptibility to suppression by Bcl-2, which was measured in NIH3T3 mouse fibroblasts. Effective metal concentrations and incubation times are given for the production of the effects listed.

another enzyme essential for apoptosis (4). This discrepancy may be explained by the fact that the latter researchers employed human T lymphocytes in which cadmium blocked apoptosis triggered by other agents.

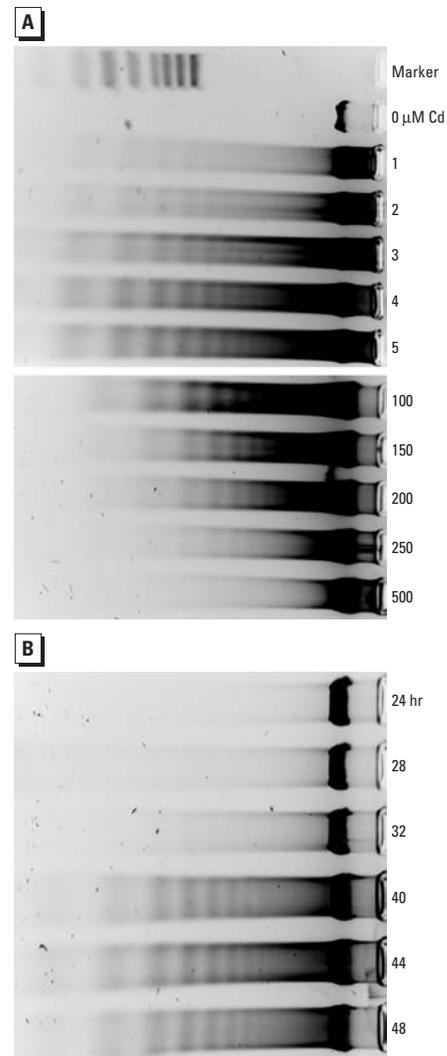
In NIH3T3 cells, apoptosis was triggered by 10 μM CdCl<sub>2</sub> within 24 hr under conditions that reduced cell viability to 50%. In these cells, overexpression of the antiapoptotic protein Bcl-2 protected against cadmium-induced apoptosis (24). This protein is known to prevent the formation of the mitochondrial permeability transition pore. Together, the observations of activation of caspase-9, the breakdown of the mitochondrial membrane potential, and the suppression of cadmium-induced apoptosis by Bcl-2 point to a mechanism whereby cadmium triggers the mitochondrial pathway of programmed cell death.

Modulators of various cellular signaling pathways were applied to investigate their possible involvement in cadmium-induced apoptosis. Programmed cell death induced by cadmium in C6 cells was found to be independent of all major signal transduction pathways investigated. It was independent of inhibition or activation of protein kinase A (PKA) and PKC; it was also independent of inhibition of MAPK, phosphatidylinositol-3-kinase, and

Ca-calmodulin-dependent protein kinase or activation of protein kinase G (3). In contrast to our observations, others have reported that the ERK (extracellular signal-regulated kinase) branch but not the p38-MAPK branch of the mitogen-activated protein kinase system was required for the induction of apoptosis by cadmium in a human T-cell line (21). This discrepancy may be due to the different cell types used. The interpretation of these data is further complicated by the observation that cadmium itself is capable of activating the formation and/or release of second messengers (inositol trisphosphate, calcium, cGMP) and stimulates some protein kinases directly or by inhibiting protein phosphatases (15,21).

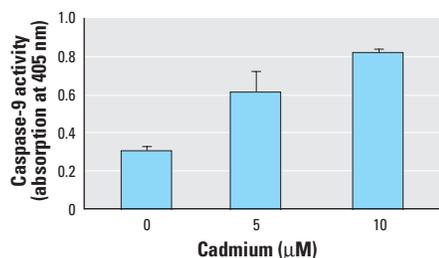
### Induction of Apoptosis by Zinc

In contrast to the solely toxic cadmium ions, zinc induces apoptosis via deficiency as well as overload. Deprivation of zinc by chelation in various mammalian cell types induced programmed cell death (11). In C6 cells, complexation of zinc by the membrane-permeable zinc chelator *N,N,N',N'*-terakis-(2-pyridylmethyl)ethylenediamine (TPEN) evoked internucleosomal DNA fragmentation (13), whereas addition of 10–50 μM ZnCl<sub>2</sub> protected the cells against DNA fragmentation evoked by 5 μM CdCl<sub>2</sub> (3). However,

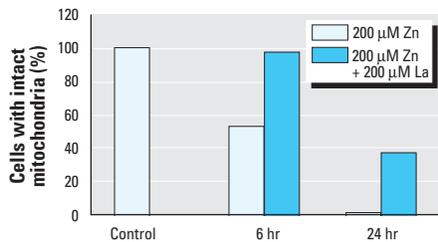


**Figure 1.** Generation of internucleosomal DNA-fragments in C6 cells by cadmium. Agarose gel electrophoresis patterns are from cells treated for 48 hr either with different concentrations of CdCl<sub>2</sub> as indicated (A) or with 10 μM CdCl<sub>2</sub> for the times shown (B). For comparison, a 100-base pair marker has been run in a separate lane in (A).

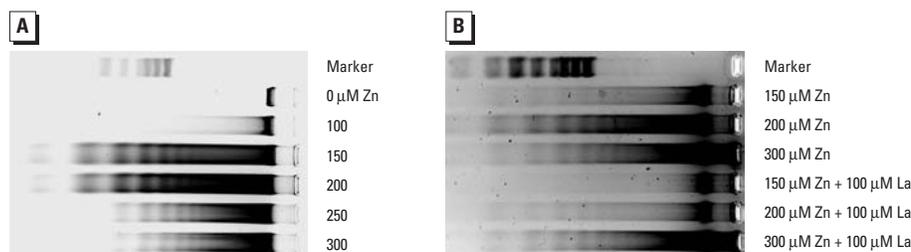
zinc at elevated concentrations also induces apoptosis with effects similar to those caused by cadmium. Zinc concentrations of 150 μM and above induced apoptosis in C6 glioma cells that involved breakdown of the mitochondrial membrane potential, chromatin condensation and nuclear fragmentation, and internucleosomal DNA fragmentation. The conditions of treatment are given in Table 2. The dependence of DNA fragmentation on the zinc concentration is shown in Figure 3A. The maximal fragmentation is observed at 200 μM of zinc, whereas at more elevated concentrations necrotic cell death seems to prevail. In a screening of eight different cell lines, zinc induced apoptotic DNA fragmentation in four cell types: C6 rat glioma cells, E367 rat neuroblastoma cells, L929 murine



**Figure 2.** Activation of caspase-9 by cadmium in C6 cells. Cells were treated with different concentrations of CdCl<sub>2</sub> for 48 hr, lysed, and incubated for 1 hr with a caspase-9–specific substrate labeled with *p*-nitrophenylacetate. Caspase activity is given in units of absorbance at 405 nm. Modified from Wätjen et al. (3).



**Figure 4.** Protective effect of LaCl<sub>3</sub> on mitochondrial damage induced by zinc. Mitochondria from cells treated with 200 μM ZnCl<sub>2</sub> for 6 and 24 hr exhibited a decreased uptake of rhodamine 123. This effect was completely prevented by the presence of 200 μM LaCl<sub>3</sub> at 6 hr and partly prevented at 24 hr of zinc treatment.



**Figure 3.** Generation of internucleosomal fragments in C6 cells by zinc. (A) Agarose gel electrophoresis of DNA from cells treated for 48 hr with different concentrations of ZnCl<sub>2</sub> as indicated. (B) DNA ladder patterns from C6 cells treated with various concentrations of ZnCl<sub>2</sub> in the presence and absence of 100 μM LaCl<sub>3</sub> for 24 hr. For comparison, a 100-base pair marker is included.

fibroblasts, and A549 human lung carcinoma cells (Table 1).

We have been interested in the effect of lanthanum(III) on zinc toxicity because the La<sup>3+</sup> ion had been shown to be an antagonist of calcium channels and of plasma membrane zinc exporters (25). Hence, we characterized the zinc-induced apoptosis by the application of lanthanum(III) chloride. Indeed, lanthanum(III) chloride at a concentration of 100 μM protected C6 cells against the DNA fragmentation induced by 150–200 μM zinc (Figure 3B) and the breakdown of the mitochondrial membrane potential (Figure 4). In this context it is remarkable that the protective lanthanum concentration was lower than the applied zinc concentration. It is surprising that lanthanum was protective, although it did not cause a decrease but an increase in the cellular zinc content as assayed by atomic absorption spectroscopy (13). Thus, the role of lanthanum in the inhibition of zinc-induced apoptosis still remains to be elucidated.

As with that of cadmium, zinc-induced apoptosis was investigated with respect to involvement of major signaling pathways. Zinc-induced apoptosis was not impaired by inhibition or activation of guanylate cyclase and PKC, or by inhibition of MAPK (13). Again, as with cadmium, the interpretation of these findings is complicated by the observations that

zinc ions modulate the activities of cyclic nucleotide phosphodiesterases (18), PKC (20), and protein phosphatases (22,23).

## Conclusions

We conclude that elevated concentrations of zinc cause programmed cell death in C6 cells in a way similar to that in cadmium, but there are also some distinct differences. For both metals, the mitochondrion seems to be the main target to trigger apoptosis. Whereas cadmium was solely toxic, zinc protected from DNA fragmentation induced by cadmium. This difference may be interpreted by the fact that zinc exerts its toxicity at a concentration that is two orders of magnitude above the effective concentration of cadmium and that at moderate concentrations it may compete with cadmium for the same cellular uptake mechanism. Furthermore, lanthanum protected against apoptosis induced by zinc but not by cadmium. Because lanthanum is known to interfere with calcium transport, the mechanism of induction of apoptosis by zinc may involve calcium mobilization, whereas cadmium acts in an independent way.

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